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Analysis of compounds in dichloromethane extractives for Sawara Falsecypress (*Chamaecyparis pisifera*) outer heartwood

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Abstract: The chemical components of dichloromethane extractives for Sawara Falsecypress heartwood were analyzed with GC/MS except for basic chemical composition analysis for heartwood with Chinese standard method. 14 kinds of compounds were identified according to the computer compounds library data. The major compounds in dichloromethane extractives comprised of terpene and naphthalene derivatives. The experiments of antifungal effects of the dichloromethane extractive on *Aspergillus niger* were also carried out. The result showed that the dichloromethane extractive from Sawara Falsecypress has no or weak antifungal capability.

Keywords: C*hamaecyparis pisifera*; Sawara Falsecypress; Outer heartwood; Dichloromethane extractives; GC/MS; Antifungal

Introduction

Sawara Falsecypress (*Chamaecyparis pisifera* (Sieb. Et Zucc.) Endl.) is derived from *Chamaecyparis formosensis* (Wang *et al*. 2003) and is an evergreen coniferous tree that grows mainly in central Japan. Sawara Falsecypress individuals grow in wet conditions on lower slopes and near mountain streams, and often occur in rocky depressions. This species not only forms mixed forests with other conifers such as *Chamaecyparis obtusa*, *Thujopsis dolabrata* and *Tsuga diversifolia*, but also mixes with deciduous broad-leaved trees, including *Fagus crenata* and *Quercus mongolica* (var. g*rosseserrata*). The life history of Sawara Falsecypress in typical old-growth mixed forests generally involves the production of seeds through sexual reproduction of mature individuals (Zhao 1988). The species plays an important role in retaining soil and sand, coast defense and wild animal protection. Cultivation of introduced Sawara Falsecypress to China started in 1936. A few years later, it was then transplanted to other provinces involving Shandong, Liaoning, Guangxi, Yunnan and the middle and lower reaches of Changjiang River. In 1968, it was relocated from Lushan botanical garden in Jiangxi province to Jiuhua farmland in Hubei province. Generally, Sawara Falsecypress is an ideal tree species with the height of 50 meter and straight trunk, whose lumber is hard and durable. Furthermore, white sapwood and brown yellow heartwood are its main characteristics. On the other hand, it is a kind of better timber for making tools, building bridge, boat, vehicle crosstie and furniture owning to its superior property of resisting decay and slinky texture. It contains abundance of cellulose which makes it outstanding material for making paper.

Sawara Falsecypress, which is famous for appreciate value, has been cultivated widely in China. The cultivation of Sawara Falsecypress is not restricted by such envirnmental factors as climate and soil, and the livability could reach as high as 90% in the production with the skewer insert method (Wu 1983; Pan 1994; Wang 1990; Zhao 1988; Lin 1987; Yu 1993; Chen 1993). At present, there are broad researches on the Cupressaceae (Chamaecyparis) in foreign countries. However, the report on the use of Chamaecyparis species in natural durability is scarce. In Japan, the wood of Chamaecyparis obtusa is evaluated for the use value of constructing important buildings such as temples and shrines and is also considered to have hygienic properties for use as counter tops in sushi bars (Koyama *et al*., 1997). Yoshixol (4,4-dimethyl-6-methylene-2-cyclohexen-1-one($C_9H_{12}O$)) extracted from several species of Chamaecyparis has been shown to possess insecticidal activity. Termiticidal activity has been reported for the heartwood of Chamaecyparis lawsoniana (McDaniel 1989) and seed extracts of this species exhibited juvenilising activity against the yellow mealworm beetle Tenebrio molitor (Jacobson *et al* 1975). Antibacterial properties have been cited for Chamaecyparis (Johnson *et al*. 2001; Xiao *et al*. 2001; Yatagai and Nakatani 1994) and Debiaggi *et al*. (1988) reported that an ethanolic extract from the leaves of Chamaecyparis lawsoniana had antiviral activity against Herpes simplex virus type 2. Some reports showed that the extractives of methyl alcohol from Sawara Falsecypress leaves had antibacterial activity against Staphylococcus aureus and obtained several antibacterial fraction.

In addition, the reports on analysis of the extractives by gas chromatograph combined with mass spectrometry (GC-MS) are limited. The alkyl- and methoxy-phenolic content in wood

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extractives were determined by the solid-phase microextraction-gas chromatography/mass spectrometry (SPME–GC/MS) (Verónica Pinon *et al.* 2007). The identification of the extractives from heartwood of Scots pine was performed using GC–MS, which gave good yields of the most important extractives: pinosylvin, pinosylvin monomethyl ether, resin acids and free fatty acids (Dag Ekeberg *et al.* 2006). The detailed composition of 70 underivated wood extractive components presenting in quaking aspen were unequivocally identified by GC-MS method, forty-four compounds by retention time and mass spectral comparison with standards. An additional 26 chromatographic peaks were assigned to broad chemical class using retention time and mass spectra features (Fernandez 2001).

In china, the studies on Sawara Falsecypress are confined itself to the growth and regeneration, young cutting from germinating shoots, growth variation and regularity, wood basic density and resource utility. (Du 1999; Zhang *et al*. 2005; Lei *et al.* 2005; Shi and Xu *et al.* 2006).

On the basis of documents retrieved, the objective of this study was to determine the basic chemical composition of outer heartwood of Sawara Falsecypress, and the antifungal activity of dichloromethane extractives against *Aspergillus niger*.

Materials and methods

Sawara Falsecypress (SF) was harvested at the eastern hillside of 850 meter above sea level, where is a gradient of 20 degree and has a good forest nutrient. Sawara Falsecypress samples were cut into three logs above ground, marked A, B and C, respectively (Fig. 1). After air-drying 2 months at the lab in Northeast Forestry University, each part log was separately cut into about twenties disks, whose thickness was 5-7 cm. In the experiment, the lowest disk of log A heartwood was cut into 4 parts depending on 5 growing rings from external heartwood to pith, then sampled correspondent part as HB-A-4, HB-A-3, HB-A-2 and HB-A-1, respectively (Fig. 2).

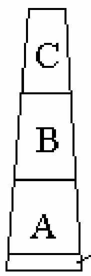

Fig. 1 Scheme of SF stem

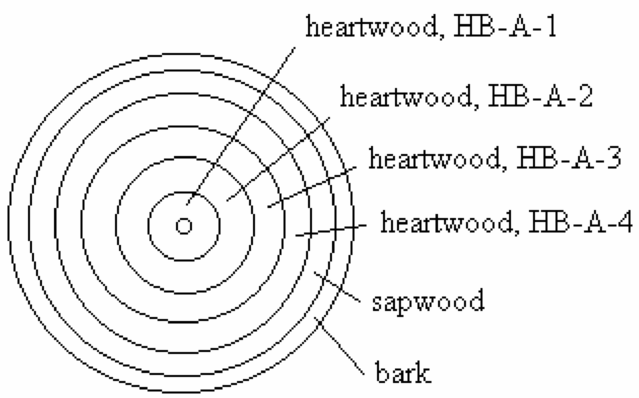

Fig. 2 Samples Scheme of Disk A

The selected sample, HB-A-4, was ground into wood powder passing through 2-mm sieves. Its moisture content (MC), cold-water and hot-water solubility, 1% sodium hydroxide solubility, alcohol-benzene solubility, nitric acid-alcohol cellulose and holocellulose, and acid-insoluble lignin content was respectively tested according to the Chinese standard method of fibrous raw material (GB/T2677.2-93; GB/T2677.4-93; GB/T2677.5-93; GB/T2677.6-94; GB/T2677.10-95; GB/T2677.8-1994) (1997). The reduplication occurred twice.

After its chemical composition analysis, the heartwood powder (HB-A-4), was extracted with hexane (n-C_6H_{14}) for 24 h, and the extracted residue was continued to be extracted with dichloromethane (CH_2Cl_2) for 24 h again (Liu *et al*. 2005). 0.04 g/ml dichloromethane extractive was tested by GC/MS 6890N-5973 insert (Agilent, USA). The instrument control parameters were listed as follows,

Method comments
DB-17 column, 30 meters, 0.25 mm ID, 0.25 micron film, FID detector.

Instrument control parameters
Injection volume: 1.0 microliters (syringe size: 10.0 microliters); spliting ratio: 1:20;
Inlet temperature: 260°C; Gas: He; Constant flow: 1ml/min; Vacuum Compensation: On

Oven program
Oven initial temperature: 40°C, Rate: 20°C /min
Second temperature: 160°C, Retention time: 6 min, Rate: 5°C /min
Final temperature: 240°C, Final time: 2 min

MS Acquisition Parameters
Link inlet temperature: 280°C, EI: 70eV; Ion source temperature: 230°C
Acquisition Mode: Scan
Scan Parameters: Low Mass: 15 amu, High Mass: 260 amu

Then the antifungal effects of the extractive of dichloromethane against *Aspergillus niger* were tested. The method was

shown as follows: each plate was injected with 0.5 mL spore solution, which was prepared after the fungal was activated, and then adding 15mL PDA media to each plate sterilized. In order to make it well-distributed, the plate should be rotated sufficiently. Then 100 μL of 0.06 g/ml dichloromethane extractive (acetone as solvent) was moved into the hole, which was made in the middle position of the plate. Compared to the one with extractive another two parallel experimental groups were assigned, one was injected with acetone and the other was blank. All the testing plates were incubated in 28°C for seven days, then observing the inhibition diameter of the fungi growth.

Results and discussion

Chemical Composition of Sawara Falsecypress

For wood powder of outer heartwood (HB-A-4), its moisture content, cold-water and hot-water solubility, 1% sodium hydroxide solubility, alcohol-benzene solubility, nitric acid-alcohol cellulose and holocellulose and acid-insluble lignin content was respectively tested according to the Chinese standard method of fibrous raw material. The result in Table 1 was the average of twice measurements. These data were the basis of further study.

Analysis of Dichloromethane Extractives Components for Sawara Falsecypress

Dichloromethane extractives of Sawara Falsecypress were tested by using GC/MS as mentioned above. Fig. 3 was the total ion chromatogram (TIC) of dichloromethane extractives for Sawara Falsecypress. 17 kinds of compounds were separated and 14 kinds of constituents were identified according to the computer compounds library data. The majority of the compounds were found. Their relative contents and match quality were listed in Table 1.

Table 1. The chemical composition of Sawara Falsecypress

Item	Heartwood (HB-A-4)	Item	Heartwood (HB-A-4)
MC%	8.60	Alcohol-benzene solubility%	4.61
Cold-water solubility%	6.60	Nitric acid-alcohol cellulose%	51.52
Hot-water solubility%	7.35	Holocellulose%	80.04
1% NaOH solubility%	15.47	Acid-insoluble lignin%	32.72

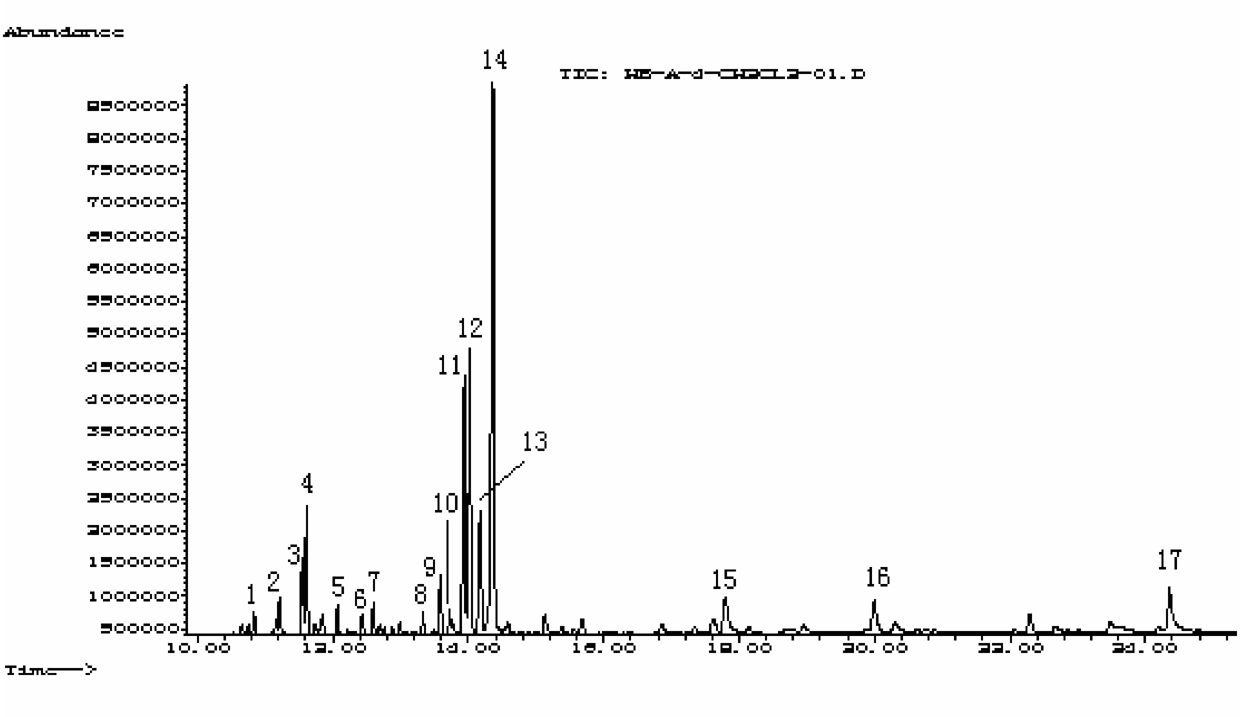

Fig. 3 Total Ion Chromatogram of dichloromethane extractives for Sawara Falsecypress

According to testing results, the major compound in dichloromethane extractives is 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-,(1.alpha,4a.alpha,8a.alpha)-naphthalene (Peak 14), its content is the highest (35.235%). But its match quality is lower, this compound need to be further identified by standard substance or other techniques such as elemental analysis and NMR analysis. The contents of Peak 11 and Peak 12 are a little lower compared with that of Peak 14, but higher than any other peaks, which their components are respective (+)-Epi-bicyclosesquiphellandrene (13.291%) and alpha-Cadinol (14.143%). Generally, 14 kinds of compounds were identified, major compounds comprised of terpenes and aromatic hydrocarbon.

According to the data in Table 1, the compounds of naphthanlene derivatives were added up to 51.54%, about half of the total extracts. Meanwhile, a little vanillin existed in the extracts.

Analysis of antifungal effects of Dichloromethane Extractives on *Aspergillus niger*

It is seen that there are no differences among the plates which the

Aspergillus niger grows as shown in Fig. 4, in other words, dichloromethane extractive has no or weak antifungal effects. According to the analysis of components in hexane extractive and dichloromethane one (Liu *et al*. 2006), both the highest contents are the same compound, namely 1,2,3,4,4a,5,6,8a- Octahydro-7-methyl-4-methylene-1-(1-methylethyl)-,(1.alpha,4a.alpha, 8a.alpha)-naphthalene. Dichloromethane extractive firstly extracted with hexane has fewer compounds than hexane one. In view of the variance, the antifungal tests in hexane extractive of Sawara Falsecypress can be carried out, then by virtue of the testing results, the antifungal components maybe exist in hexane extractive, and the antifungal components can be predicated, which is useful for further study. Furthermore, it is of great significance to choose a better isolation method in the future research for the extractive.

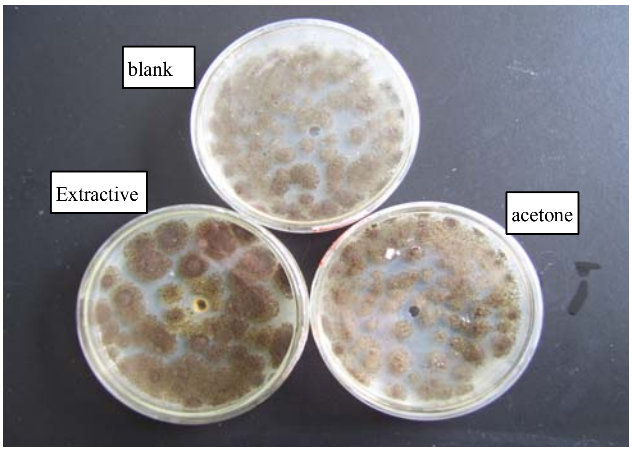

Fig. 4 Antifungal effect of dichloromethane extractive

Table 2. The assignments of peaks for the TIC of dichloromethane extractives in Fig.3.

Peak No.	Retention Time/min	Compounds	Relative Content%	Match Quality
1	10.841	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-,(1.alpha.,4a.alpha.,8a.alpha.)	0.931	99
2	11.221	unknown	1.497	–
3	11.547	Naphthalene,1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	3.568	97
4	11.616	Naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1S-cis)-	4.958	95
5	12.071	Naphthalene,1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-,(1S-cis)-	1.209	96
6	12.435	Vanillin	0.947	96
7	12.602	Benzene,1-methyl-3-[(1-methylethylidene)cyclopropyl]-	1.162	72
8	13.338	Naphthalene,1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	1.081	78
9	13.589	unknown	2.716	–
10	13.733	Naphthalene,1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	0.853	62
11	13.938	(+)-Epi-bicyclosesquiphellandrene	13.291	90
12	14.029	alpha.-Cadinol	14.143	91
13	14.181	Copaene	6.575	97
14	14.371	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-,(1.alpha.,4a.alpha.,8a.alpha.)-	35.235	60
15	17.801	Cedren-13-ol,8-	3.756	89
16	20.002	2-Naphthalenemethanol,decahydro-.alpha.,.alpha.,4a-trimethyl-8-methylene-,[2R-(2.alpha.,4a.alpha.,8a.beta.)]-	3.614	62
17	24.366	unknown	4.464	–

Conclusion

The moisture content, cold-water and hot-water solubility, 1% sodium hydroxide solubility, alcohol-benzene solubility, nitric acid-alcohol cellulose and holocellulose, and acid-insoluble lignin content was respectively tested for wood powder of Sawara Falsecypress heartwood. These data showed the basic chemical composition of Sawara Falsecypress heartwood. It is concluded that the 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methyene-1-(1-methylethyl)-, (1.alpha,4a.alpha,8a.alpha)-naphthalene is the major component of dichloromethane extractives for Sawara Falsecypress heartwood. Its relative content is the highest (35.235%), but its match quality is lower. Thus this compound needs to be further identified by standard substance or other techniques such as elemental analysis and NMR analysis. Another 2 kinds of compounds, whose contents are higher, are (+)-Epi-bicyclosesquiphellandrene (13.291%) and alpha-Cadinol (14.143%), respectively. The major compound in dichloromethane extractives comprised of terpene and naphthalene derivatives. These compounds have no antifungal capability.

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